(54) Title: COMBINATORIAL LIBRARIES HAVING A PREDETERMINED FREQUENCY OF EACH SPECIES OF TEST COMPOUND

(57) Abstract

A technique is disclosed for generating nonrandom combinatorial libraries on solid phase supports in which each of a set of predetermined species of test compounds is present on a predetermined number of solid phase supports, preferably on only one, and each solid phase support has only a single species of test compound. Each of the predetermined species of test compounds is prepared with absolute certainty because the technique does not employ any random division of the solid phase supports. Rather, the method is based on the stepwise division of a continuous solid phase support matrix prior to each synthetic step in which more than one type of subunit is added. Non-limiting examples of matrices of the solid phase supports include polypropylene membranes, polytetrafluoropropylene membranes and cotton thread. The combinatorial libraries made by the technique are also disclosed.

COMBINATORIAL LIBRARIES HAVING A PREDETERMINED FREQUENCY OF EACH SPECIES OF TEST COMPOUND.

1. FIELD OF THE INVENTION

5   The present invention concerns the field of combinatorial libraries of species of test compound that are synthesized on solid phase supports. A combinatorial library is a collection of multiple species of chemical compounds that consist of randomly selected subunits. Such libraries
10 are useful because they can be screened to identify a ligand for an acceptor of interest. More particularly the invention concerns methods for constructing such libraries when the solid phase support is a material that can be readily fashioned into further divisible pieces.

15

2. BACKGROUND OF THE INVENTION

Techniques in combinatorial chemistry are gaining wide acceptance among modern methods for the generation of new pharmaceutical leads (Gallop, M.A. et al., 1994, J. Med.
20 Chem. 37:1233-1251; Gordon, E.M. et al., 1994, J. Med. Chem. 37:1385-1401.). One combinatorial approach in use is based on a strategy involving the synthesis of libraries containing a different structure on each particle of the solid phase support, interaction of the library with a soluble receptor,
25 identification of the 'bead' which interacts with the macromolecular target, and determination of the structure carried by the identified 'bead' (Lam, K.S. et al., 1991, Nature 354:82-84). An alternative to this approach is the sequential release of defined aliquots of the compounds from
30 the solid support, with subsequent determination of activity in solution, identification of the particle from which the active compound was released, and elucidation of its structure by direct sequencing (Salmon, S.E. et al., 1993, Proc.Natl.Acad.Sci.USA 90:11708-11712), or by reading its
35 code (Kerr, J.M. et al., 1993, J.Am.Chem.Soc. 115:2529-2531; Nikolai v, V. t al., 1993, Pept. R s. 6:161-170; Ohlmeyer, M.H.J. t al., 1993, Proc.Natl.Acad.Sci.USA 90:10922-10926).

Solubl rand m combinatorial libraries can be synthsized
using a simple principle for the generation of equimolar
mixtures of peptides which was first described by Furka
(Furka, A. et al., 1988, Xth International Symposium on
5 Medicinal Chemistry, Budapest 1988; Furka, A. et al., 1988,
14th International Congress of Biochemistry, Prague 1988;
Furka, A. et al., 1991, Int. J. Peptide Protein Res. 37:487-
493). The construction of soluble libraries for iterative
screening have also been described (Houghten, R.A. et
10 al.1991, Nature 354:84-86). K.S. Lam disclosed the novel and
unexpectedly powerful technique of using insoluble random
combinatorial libraries. Lam synthesized random
combinatorial libraries on solid phase supports, so that each
support had a test compound of uniform molecular structure,
15 and screened the libraries without prior removal of the test
compounds from the support by solid phase binding protocols
(Lam, K.S. et al., 1991, Nature 354:82-84).

When random combinatorial libraries are synthesized,
however, representation of all possible structures can be
20 achieved with near certainty only in cases in which the
number of particles used for the synthesis is at least an
order of magnitude higher than the number of synthesized
structures (Burgess, K. et al., 1994, J. Med. Chem. 37:2985-
2987).

25   For library construction, it would be desirable to
perform the synthesis on a defined number of solid phase
particles exactly matching the number of compounds of which
the library is composed. This number is, generally, the
product of the number of number of types of subunits at each
30 of the predetermined number of "randomized" positions. For
example, a library of three positions (trimers) in which each
position can be occupied by one of five types of subunit has
5 x 5 x 5= 125 species. With this ideal situation, we would
be able to eliminate the statistical uncertainty of library
35 generation by the split and mix procedure. An assay of the
library might then be performed without "missing" an activ
compound or detecting the identical structure several times,

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which w uld be sp cially advantageous in the case of small
libraries (the range of tens of thousands structures), where
it is not unusual to find only single active compound (see
e.g. (Stanková, M. et al., 1994, Drug. Develop. Res. 33:146-
5 156)). Thus, there is a need in the art of synthesizing
combinatorial libraries of a method to synthesize a library
having each of the potential species of the library a
predetermined number of times, preferably once.

10   3. SUMMARY OF THE INVENTION
       The present invention encompasses a method of making a
combinatorial library on a solid phase support, the matrix of
which can be readily and continuously fashioned into separate
pieces by cutting. The test compounds of a combinatorial
15 library are comprised of subunits at various positions. The
species of test compounds of the combinatorial library are
synthesized by the stepwise addition of subunits at each
position of the test compound. The subunits are selected
from a set of types of subunits for that position. According
20 to the invention, prior to each stepwise addition, the
support matrix is divided into a number of pieces which is
equal to the number of types of subunits to be added at that
step. This process can be performed repetitively until the
piece of support matrix becomes impractically small.
25   Following the method of the invention, the practitioner
can construct a combinatorial library having a predetermined
number of species and a predetermined number of pieces of
solid phase supports. In the library there is only a single
species of test compound attached to any single piece of
30 solid support. A library constructed according to the
invention contains each of the predetermined species of test
compounds on a predetermined number of pieces of solid phase
support. In a preferred embodiment, each species will be
present on one and only one support.
35   The pres nt invention differs from th previously
employ d m th ds of synthesizing c mbinatorial libraries
becaus th m thod f th pres nt inventi n employs a

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pred termined scheme of divisi n to replac th steps of
mixing and random reassortment. These libraries are thus
termed "nonrandom" or "directed" libraries.

The methods of division can be devised so that the
5 resultant pieces are grossly recognizable, e.g., having
different lengths, widths or, in the case wherein the pieces
are planar, the pieces can be fashioned to have distinct
shapes of their sides and ends. By coordination of the
stepwise addition of the type of subunit and the gross
10 characteristics of the supports, the type of subunit can be
identified.

                4.   BRIEF DESCRIPTION OF THE FIGURES
    Figure 1: General scheme for the synthesis of directed
15 libraries. Example of the library with three positions
randomized by two amino acids in each step, generating the
library of eight tripeptides.

    Figure 2: Scheme of the synthesis of model library of 125
20 tetrapeptide mixtures on cotton string.

    Figure 3: Scheme of the synthesis of membrane library of 2888
pentapeptides. "n" = Number of amino acids randomized in the
particular position.
25
                5.   DETAILED DESCRIPTION OF THE INVENTION
        As used herein a species of test compound can be a
single defined molecular structure. Alternatively, a species
of test compound can be a motif species, which is a mixture
30 of defined molecules wherein multiple species of subunits are
present at one or more of the positions of the species of
test compound. Motif libraries are combinatorial libraries
wherein the species of test compounds are motif species. As
used herein, when motif libraries are constructed, the term
35 "type f subunit" r fers to either a chemical species of
subunit and to a defined mixture of species of subunits.
Regarding a m tif library, the p sition of a species of test

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compound wher in a mixtur of species of subunits has been
added is called a "mixed position". In keeping with the
nomeclature of random combinatorial libraries, the position
of a species of test compound of a motif library or a
5 nonmotif library wherein a single species of subunit is
present is called a "random position", except when all
species of the library have the same species of subunit at a
given position, in which case that position is called an
invariant or a fixed position. Motif libraries are described
10 in co-pending, commonly assigned patent application Serial
No. 08/246,435 filed May 20, 1994, which is hereby
incorporated by reference.

To permit the synthesis of the species of test compound
attached to the support matrix, a linking means is provided.
15 The linking means can have multiple reactive sites for
attachment of subunits. In one embodiment the subunits are
bifunctional subunits having removable protecting groups;
amino acids are the familiar example. Under these
circumstances the test compounds are polymers.
20   In an alternative embodiment the linking means comprise
multiple, orthogonally blocked reactive sites, and are termed
scaffolds. In this embodiment, the subunits provide
substituents of the scaffold. Thus, the species of test
compounds are species of substituted scaffolds. The subunit
25 added at each site of the scaffold can be bifunctional or
monofunctional and the substituent attached to a reactive
site of the scaffold can be comprised of one or more
subunits.

According to the invention the bifunctional and
30 monofunctional subunits of a library of substituted scaffolds
and the bifunctional subunits of a library of polymers can be
joined to the growing test compound by a variety of chemical
bonds of which the following are non-limiting examples:
amide, ester, urea, urethane, carbon-carbonyl bonds, carbon-
35 nitrog n b nds, carbon-carbon singl bonds, olefine bonds,
thioether bonds, and disulfid bonds. The chemistries of the
production of test compounds in scaffold and non-peptid

p lym r libraries are described in co-pending commonly
assigned patent application Serial No. 08/249,830, filed May
24, 1994, which is hereby incorporated by reference.

Solid phase synthesis of peptides is normally performed
5 on a beaded polymer (Merrifield, R.B., 1963, J.Am.Chem.Soc.
85:2149-2154), however, alternative forms of solid support
can be employed. The solid supports include membranes
(Daniels, S.B. et al., 1989, Tetrahedron Lett. 30:4345-4348),
sheets of paper (Frank, R., 1992, Tetrahedron 48:9217-9232;
10 Frank, R. and R. Döring, 1988, Tetrahedron 44:6031-6040), or
cotton (Eichler, J. and R.A. Houghten, 1993, Biochemistry
32:11035-11041; Lebl, M. and J. Eichler, 1989, Pept. Res.
2:232-235). For the review of multiple synthetic techniques
see (Frank, R., 1993, Bioorg. Med. Chem. Lett. 3:425-430;
15 Jung, G. and A.G. Beck-Sickinger, 1992, Angew. Chem. Int. Ed.
Engl. 31:367-383). Prior experience with cotton (Eichler, J.
et al., 1991, Pept. Res. 4:296-307) led to the selection of
cotton threads as one experimental solid support for the
synthesis of "directed" or "nonrandom" libraries. The
20 principle of such libraries is simple and is illustrated in
Figure 1. The continuous support is divided into as many
parts as the number of types of subunits utilized in the
first randomization step, and the pieces are individually
reacted with the appropriate residue. After completion of
25 the reaction, each fragment of the support matrix is then
divided into as many parts as the number of types of subunits
used in the second randomization step, with the appropriate
pieces from each fragment pooled for coupling.

In this manner, the synthesis can continue until
30 subunits are added at all the positions of the test compound
or until the mechanical limit of handling the support is
reached. The convenient mechanical limit of cotton thread is
achieved at a dimension of several millimeters, which defines
the practical capacity of a library of cotton thread at
35 b tween 10 test c mpounds to 50,000 test compounds.
Libraries of thes proportions are of substantial value,
esp cially in the case f nonp ptidic libraries. Thus,

librari s having as f w as two, three, four and fiv random
positions for which the number of types of subunits can be
between 2 or 3 and 19 or more are within the scope of the
invention.

5   In an alternative embodiment the matrix can be a
functionalized membranes. Non-limiting examples of a
suitable support matrix include polypropylene and
polytetratfluroethylene (TEFLON) The smallest practical area
is about 1 mm^2, and since the membrane is only 10 μm thick, a
10 library of several million compounds can be constructed most
readily when automation of this process is employed. The
size of a library constructed using a membraneous support
matrix is limited only by considerations of economy,
efficiency and the expense of screening the library and not
15 by any technical considerations of its synthesis.

A third embodiment of the invention is a "restructurable
toothbrush" having bunches of threads of functionalized
material with better mechanical properties than cotton. The
bunches of threads from different brushes can be disassembled
20 and reassembled in different combination for the next
stepwise addition of subunits. The individual bunches of
threads can be disassembled after two or three rounds of step
wise addition and recombined for the next step. For the last
randomization step the threads can be cut into pieces
25 according to the above-described method of the invention.

The disclosed method for the construction of 'directed'
libraries allows for the incorporation of simple coding
schemes, that employ simple (i.e., grossly visual)
identification of important structural features in the
30 library, based on the physical characteristics of the
support, such as the color (Campian, E. et al., 1993, 13th
APS, Edmonton, Canada) size and shape of the material (length
in the case of thread).

In constrast to libraries the species of which are
35 constructed in a fixed array, wher in the position of each
speci s identifies its structure, in the pr ferred
embodim nts of the pres nt inventi n the complete structur

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of a species of test c mp und is not encoded by a grossly
observable characteristic. Rather, the structures of the
species of test compounds selected after the screening test
are determined by structural analysis. The structural
5 analysis can be conducted by using such techniques as Edman
degradation, in the case of peptide test compounds, or mass
spectoscopy. The structure which is analyzed can be the test
compound itself or the structure can be an encoding molecule
which is attached to each solid phase support in addition to
10 the test compound. The structures of the encoding molecules
attached to a solid phase support are, firstly, arbitrarily
and systematically related to the species of the test
compound attached to the support so as to identify the test
compound and, secondly, selected so as to be readily
15 determinable. The selection and use of arbitrary coding
molecules in combinatorial libraries is described in co-
pending commonly assigned patent application Serial No.
08/249,830, filed May 24, 1994, which is hereby incorporated
by reference.
20

6. EXAMPLES

The following examples are provided as illustrative
examples of certain embodiments of the invention and are not
intended as a limitation on the scope of the present
25 invention.

We have prepared two model "nonrandom" peptide
libraries. The preparation and screening of each of these
libraries is first summarized briefly below. A more detailed
description of the materials and methods, the synthesis and
30 screening of each of these two libraries follows in Sections
6.1 - 6.5.

The first library was prepared on cotton thread and
contained only 125 peptide mixtures (see Figure 2). Cotton
thread (125 cm) was modified by beta alanine (Eichler, J. et
35 al., 1991, P pt. Res. 4:296-307) and glycine was attached to
it. The thread was then divid d into five pieces and Gly,
Ala, Leu, Ph , and Tyr w re coupled to each pi ce,

respectiv ly. Aft r c mpl te coupling and Fmoc deprotection,
a mixture of L-amino acids was coupled to every thread.
Threafter, for two successive steps, each thread fragment was
sectioned into 5 pieces and each piece was acylated with one
5   amino acid selected the above-noted set of five amino acids.
Thereafter the resultant 125 fragments were combined,
deprotected, neutralized, washed and dried; resulting in the
generation of a motif library of 125 tetrapeptide motif
species (mixtures of 19 species of peptides on each particle
10 having positions 1, 2 and 4 as random positions and position
3 as a mixed position).

      Each piece was then cut in half and both halves were
placed into two matching microtiter plates, one of them
equipped with a filter. The filter plate was then exposed to
15 ammonia gas in a desiccator to release the peptides from the
cotton. Gas ammonolytical cleavage, described earlier (Bray,
A.M. et al., 1994, J.Org.Chem. *59*:2197-2203; Bray, A.M. et
al., 1993, Tetrahedron Lett. *34*:4411-4414), was chosen as a
cleavage method because, after cleavage, the peptide remains
20 on the surface of the dry support particle. The peptides can
be dissolved into the testing buffer.

      After extraction of the peptide with buffer, the peptide
was transferred to another plate and an anti-β-endorphin
antibodies binding assay was performed. One well was
25 observed to react positively, and sequencing of the
corresponding cotton fraction, identified the sequence Tyr-
Gly-Mix-Phe (Mix is a mixture of all amino acids),
corresponding to the known motif for anti-β-endorphin
antibodies. Further analysis revealed that a one centimeter
30 of cotton thread can yield 400 nmol of released peptide,
allowing for the preparation of 40 ml of a 10 μM solution of
test compound, more than enough for multiple assays.

      The second example was a 2888 peptide library, which was
synthesized using a 16x16 cm sheet of functionalized
35 polytetraflu roethylene ("TEFLON") m mbrane. The membrane was
acylated by a N-protect d β-alanin , and a linker comp sed of
a rep at d sequence of β-alanine and glycin was constructed.

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The scheme f synth sis is given in Figur 3. Th sp ci s of
test compounds of the library had the structure Xxx-Xxx-
Pro/Gly-Gln/Phe-Phe/Leu (Xxx is one of 19 L amino acids used
for the randomization), which contained one copy of the
5 sequence Leu-His-Pro-Gln-Phe (38 copies of Xxx-His-Pro-Gln-
Xxx), with His-Pro-Gln being the known motif for
streptavidin, and one copy of Tyr-Gly-Gly-Phe-Leu, containing
Tyr-Gly-Xxx-Phe sequence, the known motif for an anti-β-
endorphin monoclonal antibody (Barrett, R.W. et al., 1992,
10 Anal. Biochem. *204*:357-364; Cwirla, S.E. et al., 1990,
Proc.Natl.Acad.Sci.USA *87*:6378-6382; Devlin, J.J. et al.,
1990, Science *249*:404-40625; Lam, K.S. et al., 1993, Bioorg.
Med. Chem. Lett. *3*:419-424; Lam, K.S. et al., 1991, Nature
354:82-84; McLafferty, M.A., 1993, Gene *128*:29-36; Pinilla,
15 C., 1993, Gene *128*:71-76). The library was then screened
with two model targets, streptavidin and anti-β-endorphin,
using the solid phase binding protocol.

Screening with streptavidin yielded 55 positive squares.
The specificity of the peptides was determined by addition of
20 the known competitor biotin. Seventeen of the squares
contained peptides that did not react with streptavidin in
the presence of biotin, however, all 17 of the squares again
stained positively in the absence of biotin with
approximately equal intensity. The peptides on three squares
25 were sequenced individually with the results given in Table
1. Small pieces of all squares were cut and placed sequenced
at once. This multiple sequencing experiment (Lebl, M. et
al., 1993, *In* C.H. Schneider and A.N. Eberle, Peptides 1992,
Proc.22.EPS Eds. ESCOM, Leiden) revealed the requirements of
30 individual positions of the peptide chain.

Incubation with anti-β-endorphin provided 21 stained
squares. These positively interacting particles were
destained, and reincubated with anti-β-endorphin antibody
using alternative detection scheme. In the first staining th
35 antibody was labelled by biotin and detecti n of b und target
was achiev d by incubation with streptavidin-alkalin
ph sphatase complex. The second round of d tection was

p rformed by incubation with anti-mouse antibody-alkalin
phosphatase complex. In this way the nonspecific interactions
of the first detection scheme were eliminated and since the
second detection was done only with selected particles, the
5 probability of detecting the nonspecific interactions
(specific interactions of the detection system) was
significantly decreased. The second incubation detected only
three positive squares. They were sequenced and sequences
found are given in Table 1. Predicted sequences with the
10 motif Tyr-Gly-__-Phe were found.

       Table 1. Sequences found on positive squares selected
for binding to streptavidin and anti-β-endorphin

15 Streptavidin   Anti-β-endorphin

   Gly-His-Pro-Gln-Phe   Tyr-Gly-Gly-Phe-Leu
   Met-His-Pro-Gln-Phe   Tyr-Gly-Gly-Phe-Phe
   Lys-His-Pro-Gln-Phe   Tyr-Gly-Pro-Phe-Leu
20
                    6.1.   MATERIALS AND METHODS
   Instrumentation :   UV/VIS absorption spectra were recorded on
   a Hewlett Packard HP 8452A Diode-Array spectrophotometer
   using a 1 cm quartz cuvette. Sequencing by Edman degradation
25 was performed on an ABI 4778 protein sequencer (Applied
   Biosystems, Foster City, CA) and Porton PI 3010 instrument
   (Porton Instruments, Tarzana, CA).
   Materials: Commercial-grade solvents were used without
   further purification. Protected amino acids were obtained
30 from Bachem (Torrance, CA), Advanced ChemTech (Louisville,
   KY), or Propeptide (Vert-le-Petit, France). Monoclonal
   anti-β-endorphin antibodies (clone 3-E7) were purchased from
   Boehringer Mannheim. Streptavidin conjugated to alkaline
   phosphatase was obtained from Pierce (Rockford, IL), and the
35 goat-anti-mouse alkaline ph sphatase compl x was obtain d
   fr m Bio-Rad (Richmond, CA). d-Biotin was purchased from
   Sigma (St. Louis, MO).

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General Procedures : S lid phase synthesis was performed
manually in polypropylene syringes (Krchňák, V. and J.
Vágner, 1990, Pept. Res. 3:182-193). Fmoc protecting groups
were cleaved with 50% piperidine/DMF for 1x10min. DIC in DMF
5 was used for the activation of Nα-Fmoc amino acids. The
completeness of each condensation reaction was monitored by
the bromophenol blue method (Krchňák, V. et al., 1988,
Collect.Czech.Chem.Commun. 53:2542-2548). The coupling
protocol included washing with DMF (6-8 times) between
10 coupling and deprotection and between deprotection and
coupling. Final deprotection was done by mixture K (King,
D.S. et al., 1990, Int. J. Peptide Protein Res. 36:255-266).

     6.2.   SYNTHESIS OF THE DIRECTED LIBRARY ON COTTON STRING
15   Cotton string (5 m, 6m/g) was treated for 1h in 25%
trifluoroacetic acid (TFA) in dichloromethane (DCM). It was
washed by DCM (3x), neutralized by 5% diisopropylethylamine
in DCM (5min) and washed by DCM and DMF (3x). Fmoc-β-Ala
(2mmol) was coupled overnight by DIC (2mmol) and HOBt (2mmol)
20 activation with addition of N-methylimidazole (3.5 mmol).
Cotton was washed by DMF and substitution was determined by
photometrical determination of cleaved Fmoc group - 0.41
mmol/g (683 nmol/cm). Five pieces of the cotton string (25
cm each) were placed into five polypropylene syringes and
25 Fmoc protected amino acids (Phe, Tyr(But), Ala, Leu, Gly)
were coupled by DIC/HOBt protocol. After coupling completion
(monitored by bromophenol blue method), the strings were
washed by DMF, placed in one syringe and Fmoc group was
cleaved. After washing with DMF and 2%solution of HOBt in DMF
30 the mixture of 19 L (cysteine excluded) Fmoc amino acids with
molar ratio (determined in pilot experiments) adjusted for
different reactivities (Eichler, J. and R.A. Houghten, 1993,
Biochemistry 32:11035-11041; Geysen, H.M. et al., 1986, Mol.
Immun. 23:709-715; Pinilla, C. et al., 1992, BioTechniques
35 13:901-905) was coupled t all cotton pi ces. After
compl ti n f this coupling the cotton was washed, Fmoc group
was cleaved and washed cotton string was divided into five

syring s in the foll wing way. From each string fiv cm of
the cotton was cut and placed in the different syringe. In
this way all syringes have only one 5cm piece of cotton cut
from each 25cm string and none have more than one. Coupling
5 of five amino acid derivatives (same as above) was performed,
cotton was washed, Fmoc group was removed and cotton was
subdivided again. In this case 1cm pieces were cut from all
5cm pieces and placed in five syringes. Coupling of the same
five amino acids was performed, Fmoc group was cleaved and
10 side chain protecting group were cleaved by mixture of TFA,
DCM and anisole (50:45:5) for 2h. Cotton pieces were washed
by DCM, MeOH and dried. Quantitative amino acid analysis of a
sample of one string revealed the substitution of 400
nmol/cm.

15

6.3.   SCREENING OF THE DIRECTED LIBRARY ON COTTON

Cotton pieces were cut in half and placed into matching
positions of the two matching microtiter plates (one equipped
with a filter bottom and one regular plate - "structure
20 evaluation plate"). Filter plate was placed into the
desiccator which was repeatedly evacuated and filled with gas
ammonia. After 20 h exposure to ammonia the desiccator was
evacuated and ammonia removed. Release buffer PBS/Tween was
added to each well (100 μl) and plates were shaken overnight
25 at room temperature. Solution was filtered to the test
plates.

For the inhibition ELISA, 96-well Immulon plates
(Dynatech) were used. Each well was initially coated with
50μl of streptavidin (20 μg/ml) in bicarbonate buffer (pH
30 9.4) (Pierce) overnight, followed by three washes with PBS.
Wells were treated with 200 μl of bovine serum albumin (BSA,
3 mg/ml) in PBS to prevent nonspecific adsorption and washed
three times with PBS/Tween, and 50 μl of biotinylated Tyr-
Gly-Gly-Phe-Leu (Salmon, S.E. et al., 1993,
35 Proc.Natl.Acad.Sci.USA *90*:11708-11712) (10 ng/ml) was added.
After 1 hr, plat s wer washed with PBS/Tween, and 50 μl of
s lution rel ased fr m c tton piec s (or Tyr-Gly-Gly-Phe-Leu,

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positiv control) was added, followed by 50 μl of anti-β-
endorphin (40 ng/ml). After 1 hr of incubation at room
temperature, plates were washed with PBS/Tween, and 50 μl of
a 1:1000 dilution of goat anti-mouse IgG horseradish
5 peroxidase conjugate (Bio-Rad) was added. One hour later, the
plates were washed, and 100 μl of solution of 30 μl of 30%
H_2O_2 in 10 ml of 2,2'-azidobis(3-ethylbenzthiazolinesulfonic
acid) substrate in citrate buffer (pH 4.2) was added. Fifteen
minutes later, the ELISA plates were read at 405 nm. Only one
10 well has shown significant inhibition. Sample of the cotton
piece (0.3mm) from the matching well in the "structure
evaluation plate" was sequenced and sequence Tyr-Gly-Mix-Phe
was found.

15   6.4.   SYNTHESIS OF THE DIRECTED LIBRARY ON FUNCTIONALIZED
                    CROSSLINKED TEFLON MEMBRANE
        Hydrophilic aminopropyl functionalized membrane (UV
crosslinked aminopropyl methacrylamide, N,N-
dimethylacrylamide and methylene-bis-acrylamide on TEFLON
20 membrane, 16 x 16 cm, Perseptive Biosystems, Bedford, MA)
with approximate 35 nmol/cm² substitution was placed into 50mL
Falcon tube and acylated by Fmoc-β-Ala using DIC/HOBt
procedure in DMF. Fmoc-Gly, Fmoc-β-Ala, and Fmoc-Gly were
coupled consecutively. After deprotection the membrane was
25 divided into two parts and Fmoc-Phe and Fmoc-Leu were coupled
to them, respectively. After coupling completion (bromophenol
blue monitoring) and deprotection, the pieces were divided
again into two halves and recombined for the coupling of
Fmoc-Gln and Fmoc-Phe. For the next coupling the membrane was
30 divided again into two pieces and recombined for coupling of
Fmoc-Pro and Fmoc-Gly. The pieces resulting from these
couplings (8 x 4 cm) were now divided into 19 strips (8 x
0.21 cm) and the strips were placed into 19 small
polypropylene tubes. Nineteen natural amino acids (excluding
35 Cys) were used for coupling in this stage. After coupling
compl ti n and Fmoc deprotection, The strips w r cut into 19
pieces (4 x 2.1 mm) and divided into 19 vessels again. Th

sam s t of 19 Fmoc amino acids was used for the last
coupling. All pieces were combined, Fmoc group was removed
and side chain protecting groups were cleaved by mixture K
(King, D.S. et al., 1990, Int. J. Peptide Protein Res.
5 *36*:255-266). Membrane pieces were washed by TFA (2X), DCM
(5X), MeOH (3X), water (5X). Substitution based on
measurement of the absorbance of the last Fmoc release was
43.3 nmol/cm^2.

10 6.5.   SCREENING OF THE DIRECTED LIBRARY ON CROSSLINKED TEFLON
MEMBRANE

    The peptide library was screened according to published
protocol (Lam, K.S. and M. Lebl, 1992, Immunomethods *1*:11-
15). The peptide squares were first washed with double-
15 distilled water. After thorough washing, the squares were
washed and coated with 0.05% gelatin (w/v) to block
nonspecific binding. Washing with PBS/Tween (137 mM NaCl,
2.7 mM KCl, 4.3 Na$_2$HPO$_4$, 1.4mM KH$_2$PO$_4$, pH 7.2 with 0.1% Tween-
20) and 2xPBS/Tween/gelatin (2x PBS, 0.1% Tween-20 (v/v),
20 and 0.05% gelatin (w/v)) was followed by an incubation with a
20nM streptavidin-alkaline phosphatase in 2x
PBS/Tween/gelatin. Again the library was washed with
PBS/Tween, 2xPBS/Tween/gelatin and with TBS (137mM NaCl,
2.7mM KCl, 25mM Tris base, pH7.4). The standard substrate 5-
25 bromo-4-chloro-3-indolyl phosphate was added then added. The
library and substrate were then transferred to petri dishes
for color development. Fifty-five color squares were
collected, washed with 6M guanidine hydrochloride, pH 1.0,
decolorized with DMF, gelatin coated and competed with 100nM
30 d-Biotin with 20nM steptavidin-AP in 2xPBS/Tween/gelatin.
Incubation with substrate yielded 17 colorless, competed
squares. Reincubation with 20nM streptavidin-AP produced 17
positively reacting squares following treatment with
substrate.
35   Th remaining library was then r cycl d with 6M
guanidin hydrochlorid , pH1.0, DMF, and gelatin coated.
Aft r washing with PBS/Tw en and 2xPBS/Twe n/gelatin,

250ng/ml biotinylated anti-β-end rphin antibody in
2xPBS/Tween/gelatin was added.   Following thorough washing,
streptavidin-alkaline phosphatase was added.   The library was
then washed, substrate was added, and color development
5 proceeded as described above.   Twenty one squares developed
color, and subsequent recycling prepared them for specificity
determination.   Using 2xPBS/Tween/gelatin as the buffer,
200ng/ml anti-β-endorphin antibody was added, and thorough
washing was followed with 1.5nM goat-anti-mouse-AP.   The
10 squares were then washed with PBS/Tween, 2xPBS/Tween/gelatin
and TBS, and substrate was added.   As a result, 3 squares
developed the color that indicates binding.

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I CLAIM:

1.   A combinatorial library of multiple species of test compounds comprising:

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      a)   a predetermined number of separate solid phase supports; each support consisting of
            i.   a single piece of a readily and continuously divisible matrix, and
10   ii.   a covalent linking means; and
      b)   a predetermined number of predefined species of test compounds attached to the supports through the linking means; each species comprising a predetermined number of subunits, said subunits
15   each occupying a predefined position;
   in which each species of test compound is attached to a predetermined number of supports and each support is attached to a single species of test compound.

20 2.   The library of claim 1 in which for every species of test compound, the identity of the type of subunit at a first position is independent of the identity of the type of subunit at a second position.

25 3.   The library of claim 1 in which a grossly observable characteristic of the solid phase support identifies a type of subunit at a position of a species of test compound attached to the support.

30 4.   The library of claim 3 in which the grossly observable characteristic is the size of the support.

5.   The library of claim 3 in which the grossly observable characteristic is the shape of the support.

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6.   The library of claim 3 in which the grossly observable characteristic is the c lor of th support.

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7.   The library of claim 1 in which every position f the
species of test compounds is a random position or an
invariant position.

5 8.   The library of claim 1 in which the species of test
compounds are motif species.

9.   The library of claim 1 in which the species of test
compounds are polymers.
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10.   The library of claim 1 in which the linking means
comprises a plurality of orthogonally blocked sites of
attachment.

15 11.   The library of claim 1 in which the subunits of the test
compounds are linked by chemical bonds selected from the
group consisting of amide, ester, urea, urethane, carbon-
carbonyl bonds, carbon-nitrogen bonds, carbon-carbon single
bonds, olefine bonds, thioether bonds, and disulfide bonds.
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12.   The library of claim 1 in which each type of subunit
comprises an amino moiety and a carboxyl moiety.

13.   The library of claim 1 which further comprises multipl
25 species of encoding molecules, wherein the species of coding
molecules attached to a solid phase support identify a
species of test compound attached to the support.

14.   A method of making a combinatorial library which
30 comprises at least two successive iterations of the steps of:
        a)   selecting a set consisting of two or more types of
              subunits;
        b)   dividing a continuously divisible solid phase
              support having reactive sites into a number of
35   pi ces equal to the number of sel cted typ s f
              subunits;

c)   attaching one type of subunit t substantially all
the reactive sites of a corresponding piece of
solid phase support; and thereafter

d)   removing a protecting group so that reactive sites
5   are provided.

15.   The method of claim 14 wherein the protecting group is a
type of protecting group of a linker of the solid phase
support, said linker having a multiplicity of types of
10 orthogonal protecting groups.

16.   The method of claim 14 wherein the subunit is a
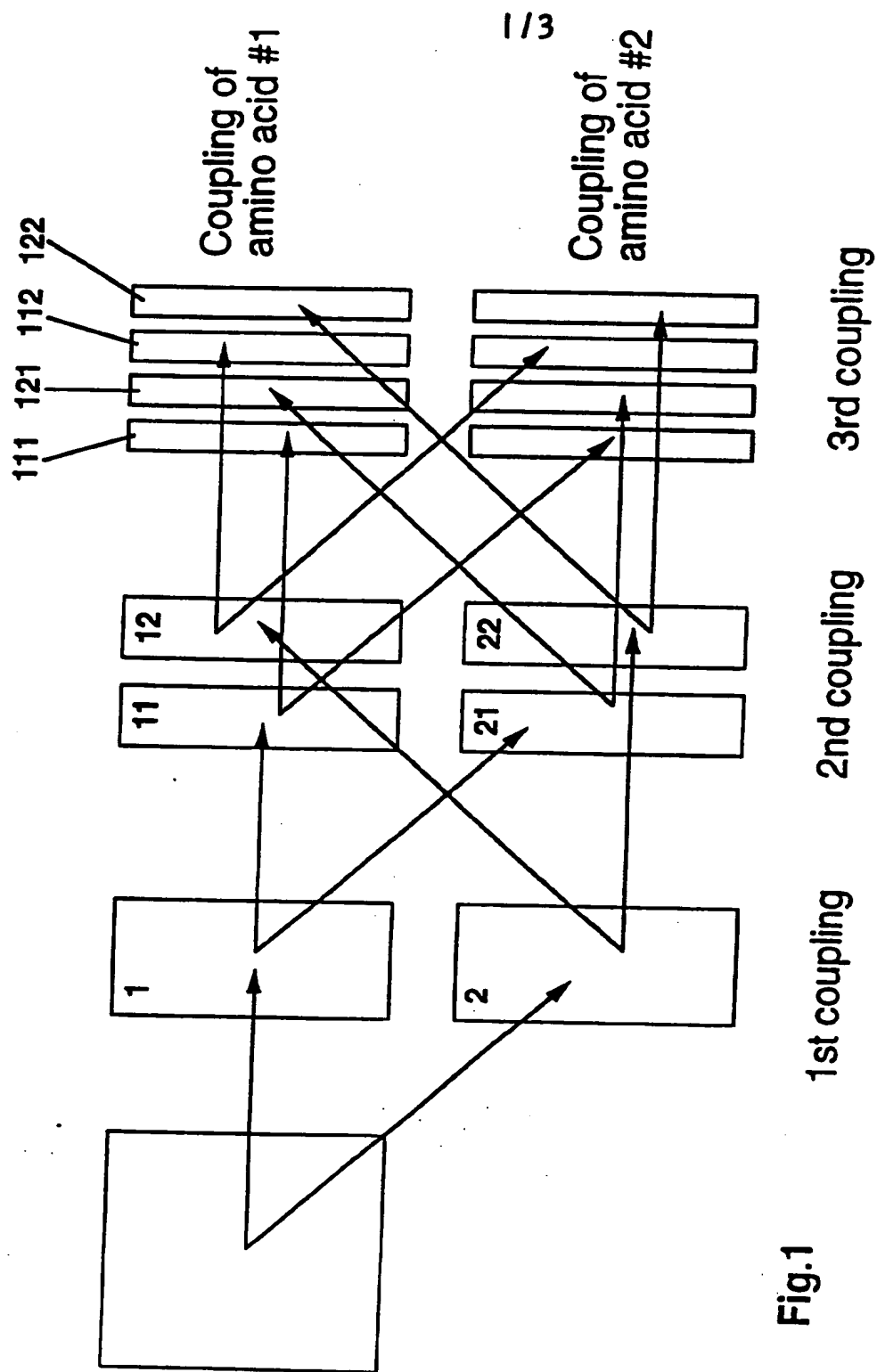bifunctional subunit and the protecting group is a
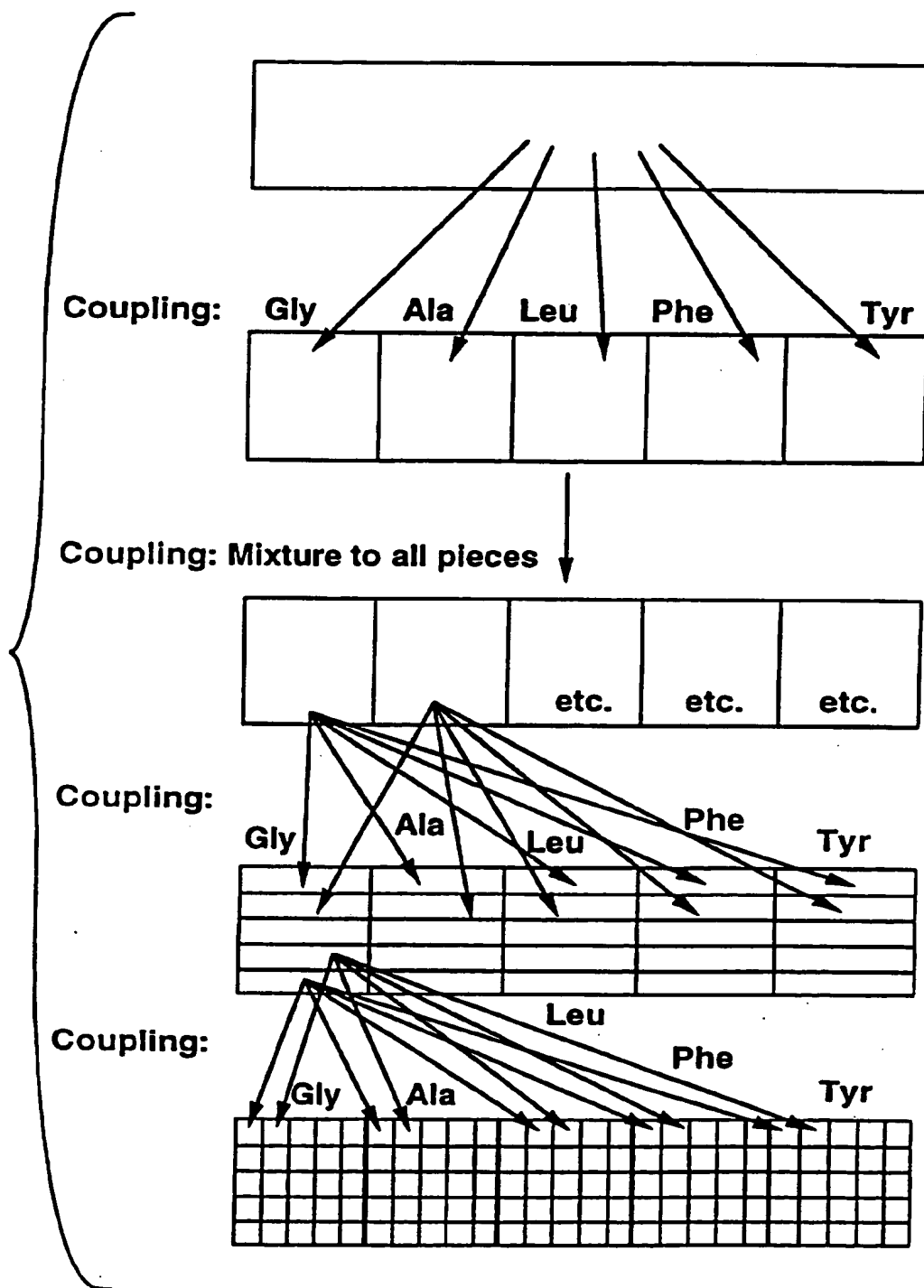substituent of the subunit.

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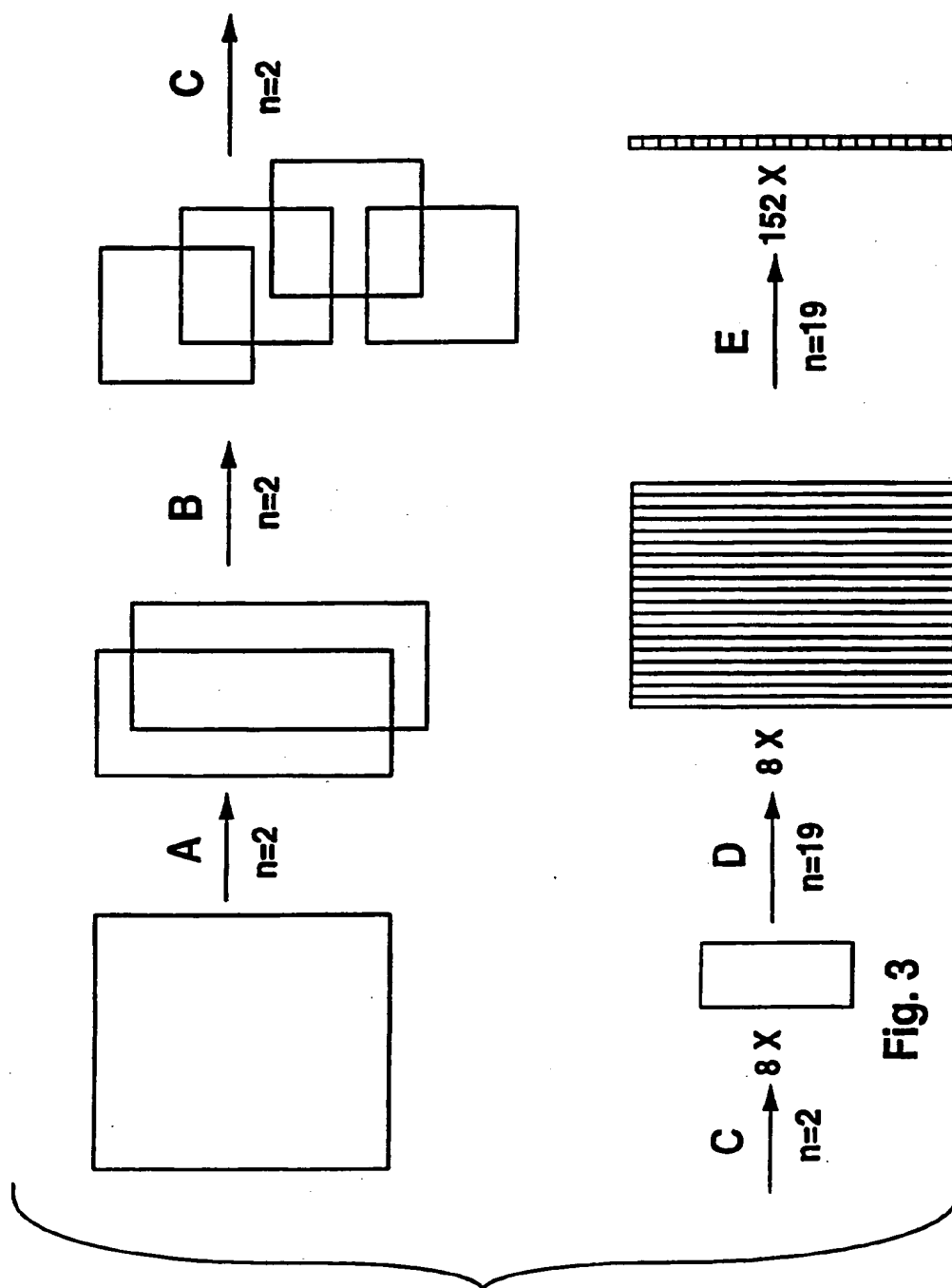
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Fig.1

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Fig. 2

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Fig. 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543
US CL :436/518

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 501, 531; 435/7.1; 530/333, 334

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS
search terms: peptide, combinatorial, library, color, shape, visible

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A, 92/00091 (BIOLIGAND, INC.) 09 January 1992, pages 9, 10, 26-28.	1, 2, 7-12, 14-16
X	WO, A, 93/24517 (FURKA ET AL.) 09 December 1993, see pages 6-8, 10 and 11.	1-16

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 FEBRUARY 1996	12 MAR 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*